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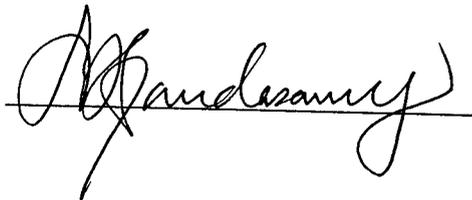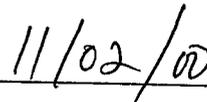
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The purpose of this two-year IDEA proposal is to gather preliminary data that demonstrate the feasibility of a new class of *in vitro* drug response and chemosensitivity assays. Our objectives are to develop an instrument (a visual servoing optical microscope, or VSOM) in a stepwise fashion and gather preliminary data to aid future development of cell culture techniques and fluorescence assays. Our goal is to demonstrate the feasibility of using VSOM technology to analyze the heterogeneity and physiological characteristics of tumor cells from individual breast cancer patient biopsies. We believe that the VSOM will be extremely useful in developing new VSOM chemosensitivity fluorescence assays for a variety of breast cancer drugs. At the conclusion of this grant, we will have preliminary data that demonstrate the use of a VSOM doxorubicin chemosensitivity assay based on the dynamic physiological responses of single cells.

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FOREWORD

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Samuel E. Callahan 9/29/99
PI - Signature   Date

Table of Contents

Introduction

The purpose of this two-year IDEA proposal is to gather preliminary data that demonstrate the feasibility of a new class of *in vitro* drug response and chemosensitivity assays. Our objectives are to develop an instrument (a visual servoing optical microscope, or VSOM) in a stepwise fashion and gather preliminary data to aid future development of cell culture techniques and fluorescence assays. Our goal is to demonstrate the feasibility of using VSOM technology to analyze the heterogeneity and physiological characteristics of tumor cells from individual breast cancer patient biopsies. We believe that the VSOM will be extremely useful in developing new VSOM chemosensitivity fluorescence assays for a variety of breast cancer drugs. At the conclusion of this grant, we will have preliminary data that demonstrate the use of a VSOM doxorubicin chemosensitivity assay based on the dynamic physiological responses of single cells.

Body

Technical Objective 1: Development of an Automated VSOM System

Task 1: Develop a prototype, non-automated VSOM system.

We have completed the integration of hardware components necessary for elementary and advanced Visual Servoing Optical Microscopy. As seen in **Figure 1 (C,D, and E)**, we have modified an open perfusion microincubator so that it couples securely with a computer controlled x,y scanning stage on an inverted optical microscope. We have also obtained two computer-controlled perfusion pumps (**Figures 2A and B**) allowing us to precisely control the time, duration and rate of perfusion from four 50 mL syringes. A vacuum aspirator removes excess liquid from the chamber during perfusion experiments (**Figure 1E, right arrow**).

The following progress has been made on system integration and software development. of the VSOM system. The target instrument is an inverted optical microscope. We have developed a plugin that interfaces this instrument to DeepView. [1,2] DeepView is a channel for distributed microscopy and informatic, and it is funded through the DOE National Collaboratory program. Deepview provides a scalable interface for adding any instrument into its framework. It also provides the means for data transfer and viewing over the wide area network. The system has a generic graphical user interface (or GUI, **Figure 3**) that interacts with the target instrument through advertisement of its properties. In the case of VSOM, related properties are computer controlled syringes, shutters, a filter wheel, and camera control parameters. This concept of advertising capabilities (functionalities) of an instrument leads to a more uniform interface and reduces maintenance cost through software reusibility. One way to achieve this is through implementing a plugin, which has been accomplished for VSOM. The system allows the user to enter a recipe for a particular experiment. The recipe allows for (a) controlling the perfusion rate into the cell chamber, (b) setting camera exposure times, (c) selecting the location of the filter wheel, and (d) setting the sampling rate for data collection. Once the data is collected, they are then processed for aggregating the cell responses. DeepView provides a particular service for image analysis. It is a collection of class and class libraries for quantitative analysis. Our system segments (localizes) each nucleus using adaptive Hough transform in the blue channel. Once that these cells are found, their responses in the green and red channels are measured and tabulated. The entire operation is automatic to allow large scale population studies. Segmentation of cells from the transmission light has been a a complex problem. We have developed an algorithm to

segment images with similar characteristic through the grouping principal.[3] Our approach is to extract partial cues corresponding to step and roof edges of the image and then group them through geometric reasoning. Step edges form the boundary between the nuclei and background, and roof edges provide the partial boundary between adjacent nuclei. The intent is to construct a partitioning that is globally consistent. A unique feature of this system is in hyperquadric representation of each hypothesis and the use of this representation for global consistency. The main advantage of such a parameterized representation is compactness and better stability in shape description from partial information. Presently, this approach is computationally expensive and lacks the required automation for on-line analysis. We will continue to refine and update this approach, however, our primary focus will be in closing the loop between cell responses and flow control to meet the visual servoing objectives.

In summary, we have achieved semi-automated, computer control of the transmitted and fluorescence light shutters, the optical filter wheel, both perfusion pumps (4 independently controlled syringes), the z-axis focusing motor, the xy stage, and the digital camera . During a typical experiment, the fluorescence and transmitted light exposure times and filter wheel positions are specified, as is the duration of the experiment and the interval between image acquisitions. A perfusion schedule is also specified. This schedule lists, for each syringe, the perfusion start time, the flow rate, and the perfusion stop time. At each image acquisition interval four dark current corrected digital images are acquired (1024x1024 pixels). In data presented here, the blue fluorescence image is acquired using 360nm excitation light (filter wheel position 1), the green image is acquired using 490nm excitation light (filter wheel position 2), the red image is acquired using 570nm excitation light (filter wheel position 3), and the bright field transmitted light image of the cells is acquired using transmitted light. A 10X Zeiss Fluar objective, NA 0.5 was used for image acquisition. The area observed in one 1024x1024 image at 10X magnification is 717 microns by 717 microns. Typically 200-500 individual living cells are observed in a stationary, single field of view.

Task 2 Analyze image data off-line.

Images are currently processed off-line. For initial, qualitative visual inspection of a particular experiment, all 12-bit images (0-4095 gray levels) are reduced to 8-bit tif format images using a different, carefully chosen scale for each channel. Ordered, time-lapse contact sheets are constructed for each separate channel, each channel is assigned an artificial color (blue, green, or red). These three sets of monochrome (b/w) images are then combined into a single set of time-lapse color images. **Figures 4 and 5** are examples of such contact sheets. The scaled, full-color tiff images are also assembled into time-lapse, animated digital movies. In some cases the transmitted light images are then overlaid on the multicolored fluorescence light images as demonstrated in **Figure 6**.

Cell responses are quantified, and reduced data is visualized, in the following manner. Cells are segmented using the software described above. The stained nuclei of living cells are detected in the blue channel images. Our eventual goal is to segment images using the transmitted light image only. At the present time, nuclear staining is providing valuable information and is an important aid to developing our transmitted light segmentation algorithm. **Figures 3 and 7** are examples of cell segmentation, labeling, and tracking using our software. The mean intensity of each circular region of interest (ROI) is computed for each of the three fluorescence channels (r,g, and b). Responses are plotted per channel as individual cell responses (shown schematically in **Figure 8**) and as a mean response for all cells, ± standard deviation (**Figure 9**, actual experimental data).

Technical Objective 2: Development of VSOM Fluorescence Assays

Tasks 6 and 7: Perform manual mode VSOM experiments on different cell lines

The following cell types have been examined: MCF-7 (human breast cancer cell line, drug sensitive , or DS), MCF-7adr (multi-drug resistant , or MDR, version of MCF-7), 184SK (normal HMECs), 184B5 (immortally transformed HMECs), AaZ2', and B5KTu (tumorigenically transformed HMECs).

A new, dedicated cell culture room (74-176A) across the hall from the LBNL Life SciencesMicroscope Resource (74-174) has been established and is now in operation (**Figures 1 A, B**). Currently, this room is used soley by Dr. Callahan for cell culture work supporting this DoD grant (DAMD17-98-1-8177). Perfusion experiments have been conducted using a nuclear stain (Hoechst 33342, blue chanel) an acidotropic, lysosomal stain (Lysotracker Red, red channel), and a cytoplasmic, cell viability stain (calcein-AM, green channel). There have been literature reports that both Hoechst 33342 and calcein-AM are actively extruded from MDR cells by an energy dependent efflux mechanism.[1,2] Thus, these cells are expected to be less fluorescent than DS cells in the blue and green channel. However, some MCF-7adr MDR cell lines have been shown to have internal vesicles that are more acidic than DS cells.[3] In this case, MDR cells stained with Lysotracker Red, a dye that accumulates in low pH cell compartments, would be expected to be more fluorescent in the red channel.

We have obtained MCF-7 (DS) and an MCF-7adr (MDR) cells lines from a researcher in our Division, Dr. Ruth Lupu. We have not yet verified reduced doxorubicin cytotoxicity in these MCF-7 adr cells. **Figure 10** is a schematic of our perfusion experiments. This schematic may be helpful in understanding the large amount of data plotted in **Figure 9**; however, there are some slight differences between the experiments depicted in **Figures 9 and 10**.

As seen in **Figure 9**, we observed very little difference between the DS (MCF-7) and MDR (MCF-7adr) cells. This was true for three independent fluorescence assays for MDR: Hoechst 33342, Calcein-AM, and Lysotracker Red (LT-R). It is possible that the MCF-7adr cells we received have reverted to the DS phenotype. We plan to check for the MDR phenotype by performing cytotoxicity assays with doxorubicin, and we have also contacted other researchers about obtaining a different matched pair of MCF-7 and MCF-7adr cell lines.

The buffer used in obtaining the data shown in **Figure 9** was Dulbecco' s Phosphate Buffered Saline, (D-PBS) containing normal concentrations of calcium and magnesium. It also contained glucose (1000 mg/L) and sodium pyruvate (36 mg/L) without phenol red (GibcoBRL, #14287-080). We have noted that the composition of the buffer (D-PBS without calcium, magnesium, glucose , or pyruvate) has a large effect on calcein retention in MCF-7 cells. If media containing serum and/or phenol red are present during buffer exchange, the phenol red appears to act as a photosensitizer, and cells are rapidly photobeached. These experimental parameters will be closely observed during our Year 2 experiments.

Key Research Accomplishments

- Integration of VSOM Hardware and Software

- Establishment of Dedicated Tissue Culture Facility near the VSOM

- Time-Lapse Video of Hundreds of Living Cells during Multiple Perfusions

- Observation of Different Physiological Responses in Four Separate Channels

- Data Reduction and Display of Hundreds of Single Cell Responses in 3 Channels

Reportable Outcomes

LBNL Patent Disclosure: Callahan, D.E., and Parvin, B."A Method for Knowledge-Based Discovery and Optimization of Differences Between Cell Types" September 9, 1999.

Additional Funding Sought: Parvin, B., Callahan, D. and Cong, G. "Visual Servoing for Knowledge-Based Interrogation of Living Human Cells" Department of Energy Announcement LAB99-18, *Pilot Projects in Biomedical Engineering*, May 1999. <u>NOT FUNDED.</u>

Conclusions

VSOM fluorescence assays have been very successful up to this point, because the cells remain viable at room temperature for 5-6 hrs on the microscope stage, and we can continously observe them in four channels as we perfuse fluorescent compounds into the chamber. We have the infrastructure in place to handle very large amounts of digital video data and perform on-line analysis and intelligent instrument control. Once the VSOM is fully automated it will be extremely useful for dissecting complex biochemical pathways, isolating and optimizing important environmental variables, and designing fluorescence assays for specific cell types or for breast cancer drug chemosensitivity..

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Appendix A: FigureLegends

Figure 1: **(A,B)** Cell Culture Facilities **(C-E)** Modifications of cell chamber and x,y scanning stage.

Figure 2: **(A,B)** VSOM with computer controlled syringes. **(C,D)** Triple-stained cells at different times during a time-lapse perfusion experiment **(E-G)** Cells stained with Hoechst, Calcein-AM, and LysoTracker Red, respectively and then overlaid with a transmitted light image.

Figure 3: Deep View Graphical User Interface (three views).

Figure 4: Time-Lapse images of the blue and green channels.

Figure 5: Time-Lapse images of the red channel and combined color images.

Figure 6: Four Channel Image showing red, green, and blue fluorescence overlaid with a transmitted light image.

Figure 7: Outlined cell nuclei for image segmentation and analysis

Figure 8: Schematic example of Two Single cell responses from the blue channel.

Figure 9: Three channel mean responses of all MCF-7 (DS) cells overlaid all the three channel mean responses of all MCF-7adr (MDR) cells.

Figure 10: Schematic of a VSOM perfusion experiment.

Appendix B: Figures
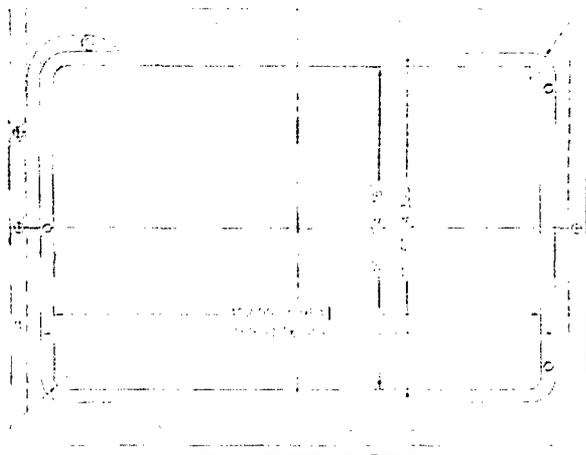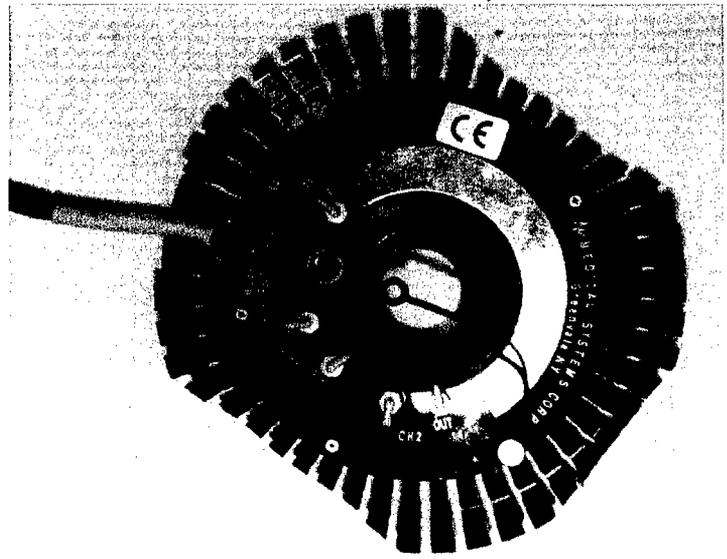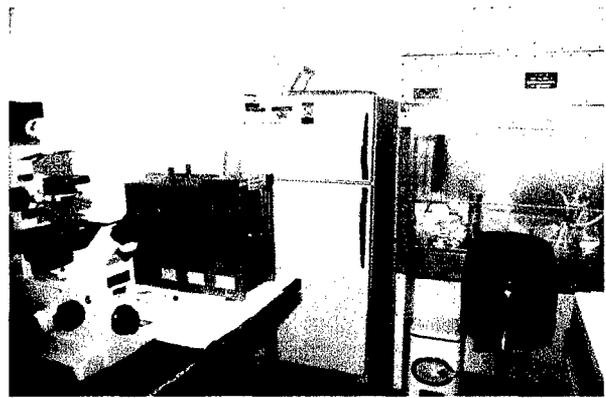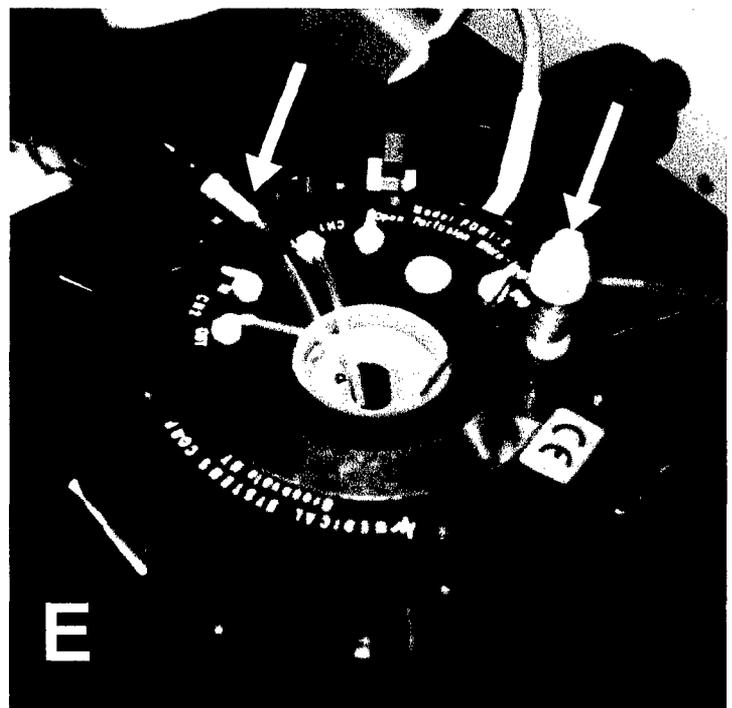
FIGURE 1

FIGURE 2

FIGURE 3

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DS, MCF-7, Blue 250-200, Green 0-500, Red 30-1000

FIGURE 4

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DS, MCF-7, Blue 250-200, Green 0-500, Red 30-1000
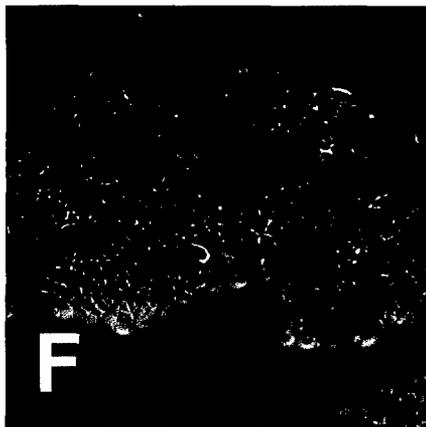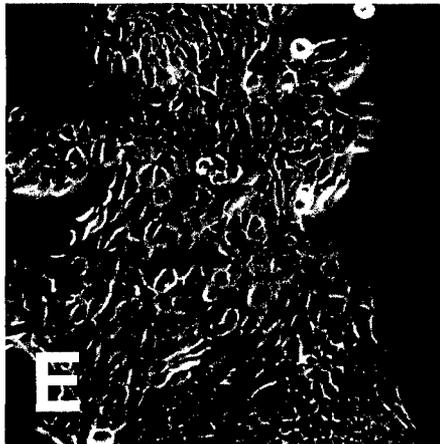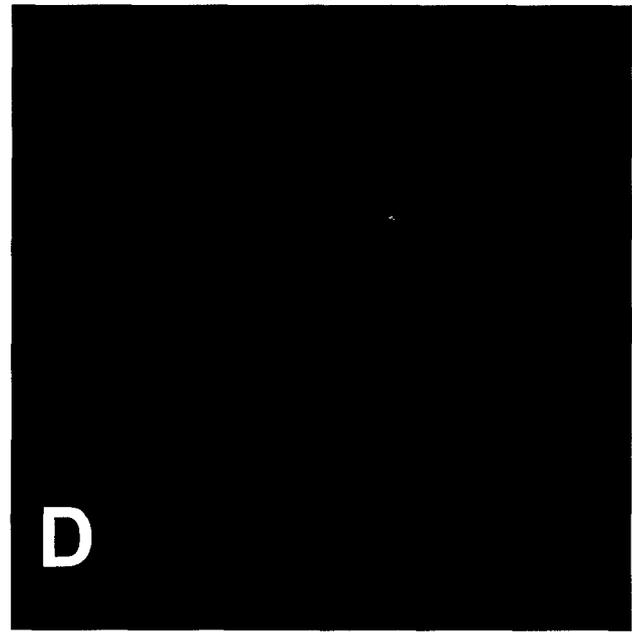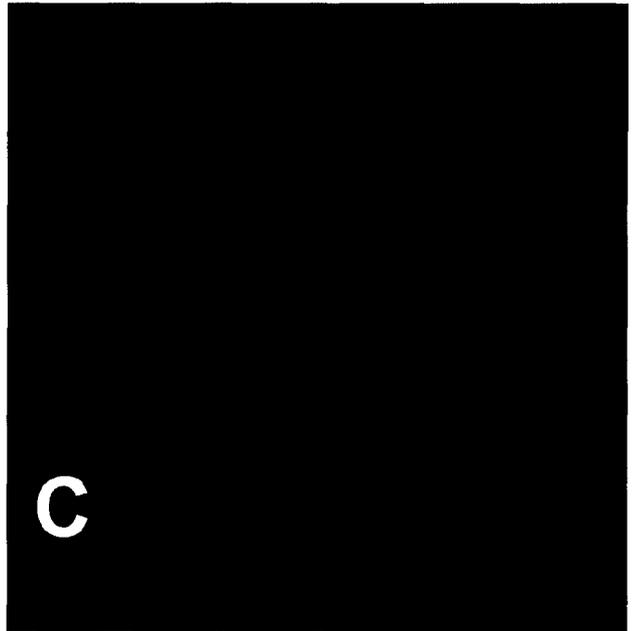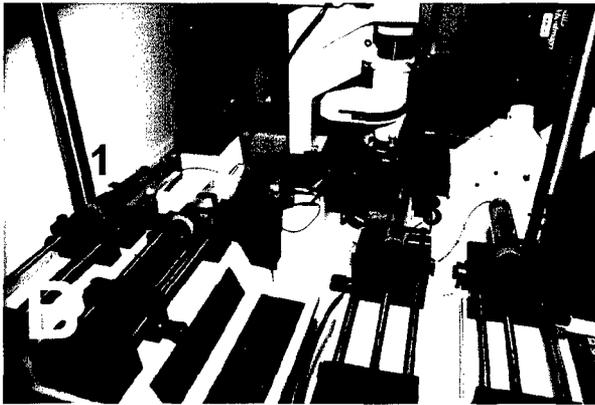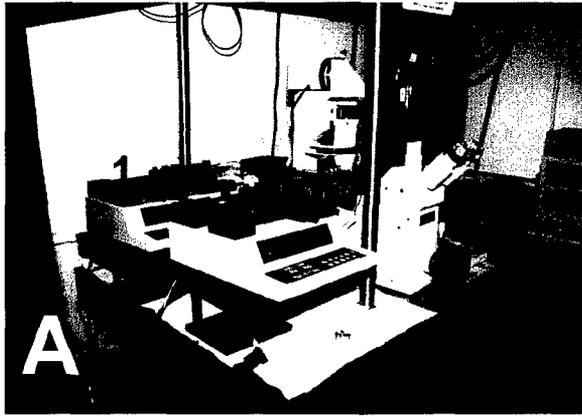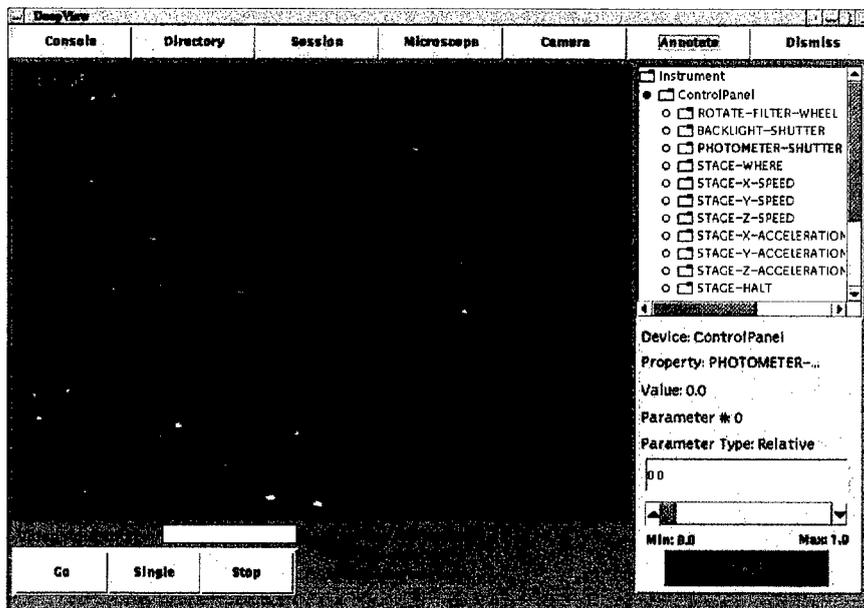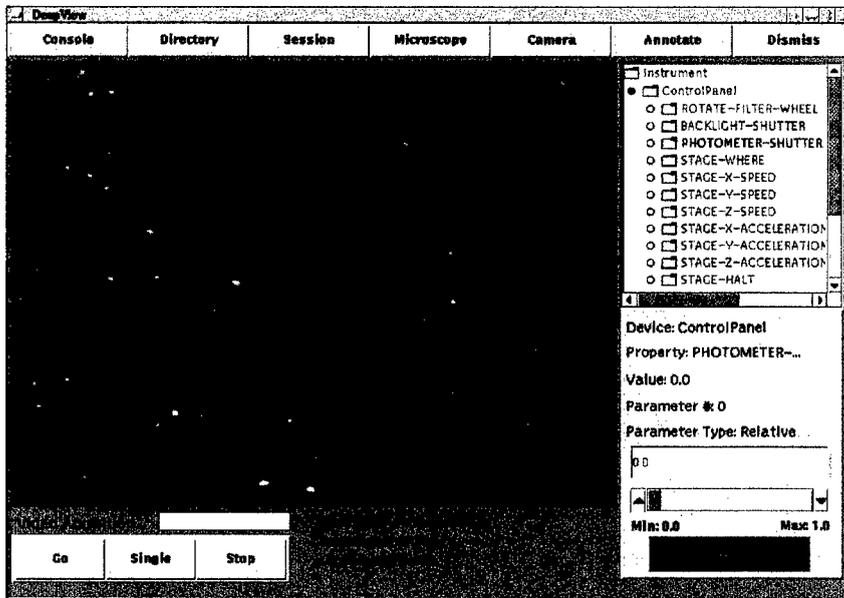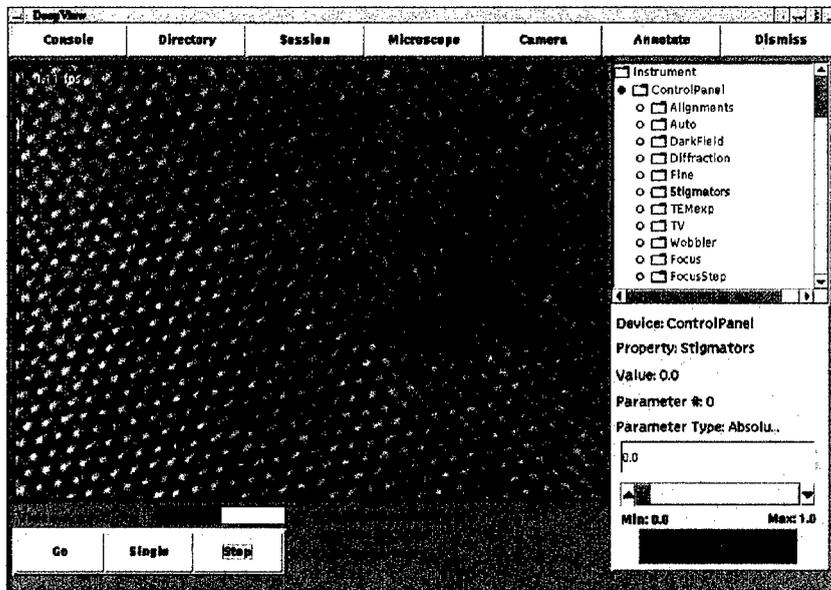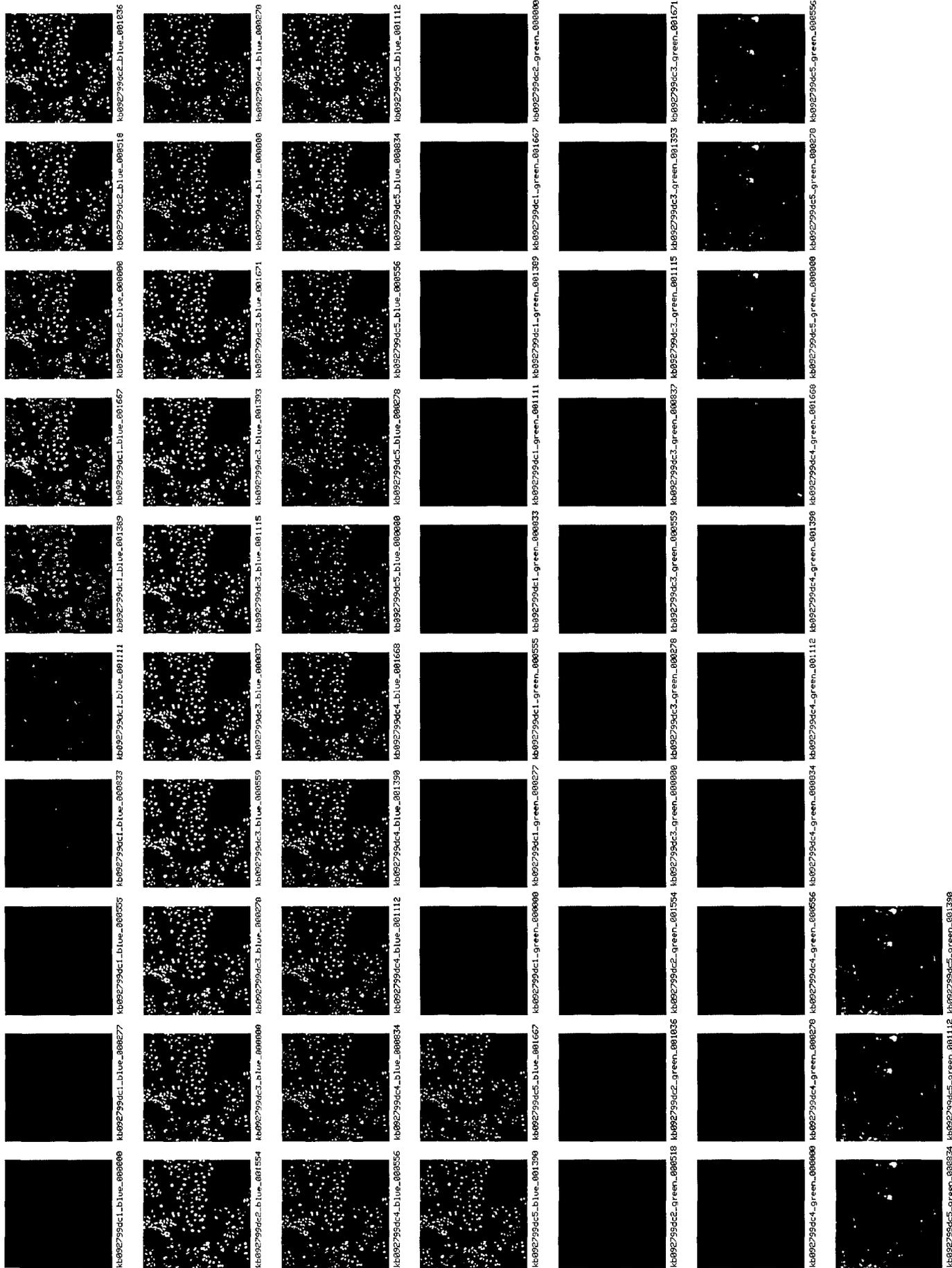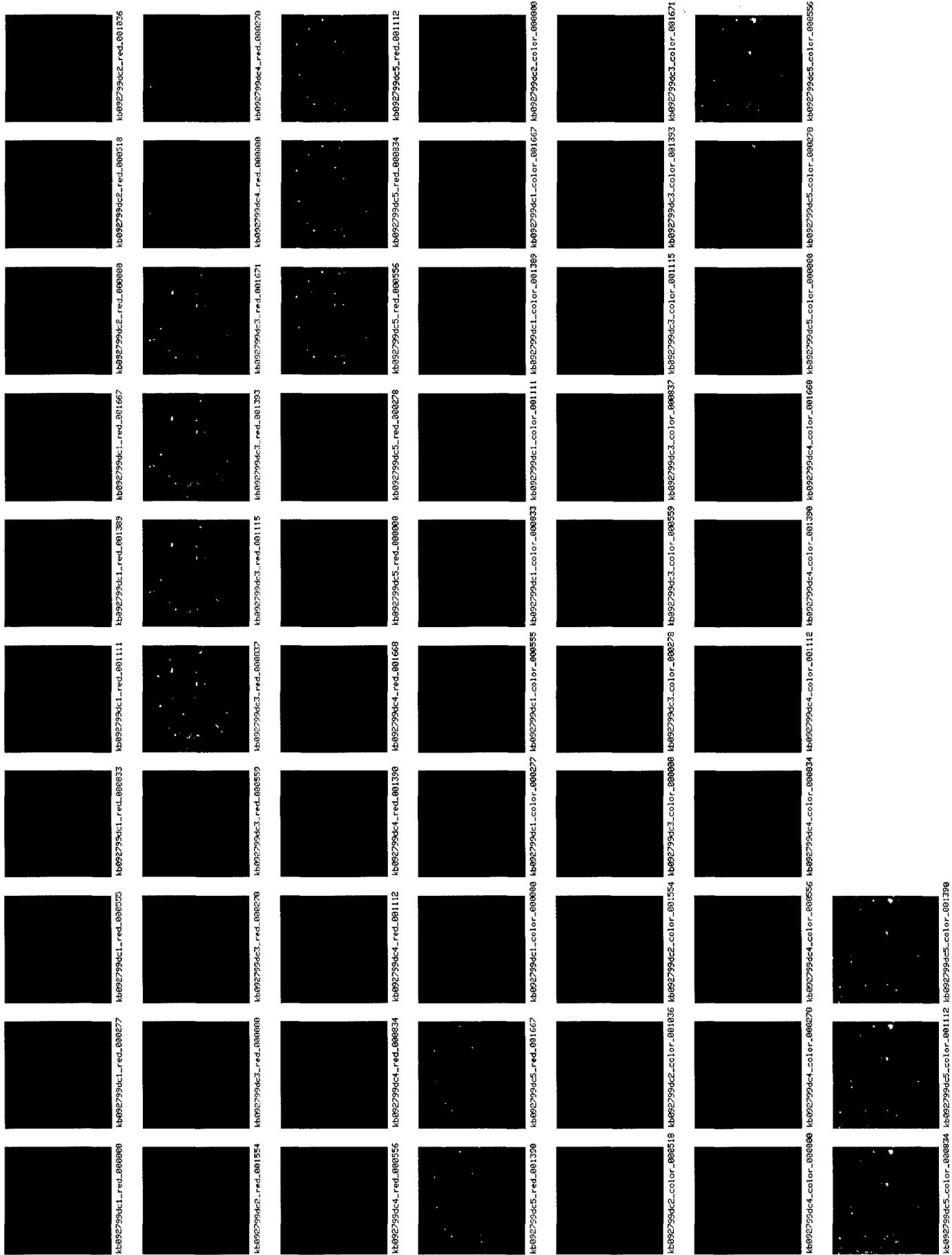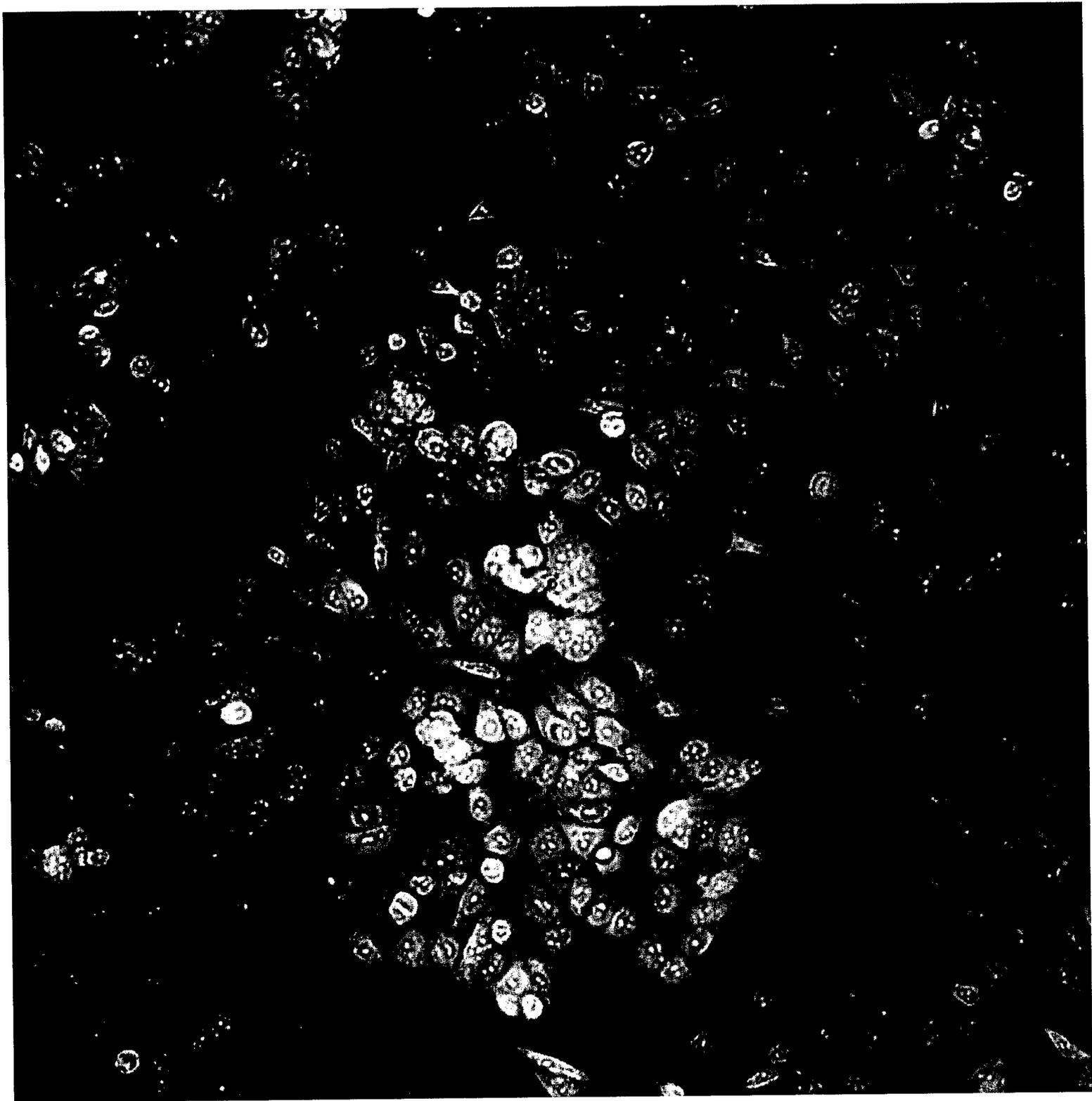

FIGURE 5

FIGURE 6

FIGURE 7

Example of Two Single Cell Responses Monitored in the Blue Channel

Hundreds of Single Cell Responses are Regularly Obtained in one Experiment. The Uptake of a Nuclear Stain Can be Monitored in the Blue Channel, While the Uptake of a Lysosomal Stain (LysoTracker Red) is being Monitored in the Red Channel (not shown). At the same time, the Uptake of a Cytoplasmic Dye (Calcein AM) is Monitored in the Green Channel, and Changes in Morphology are Tracked in the Transmitted Light Channel.
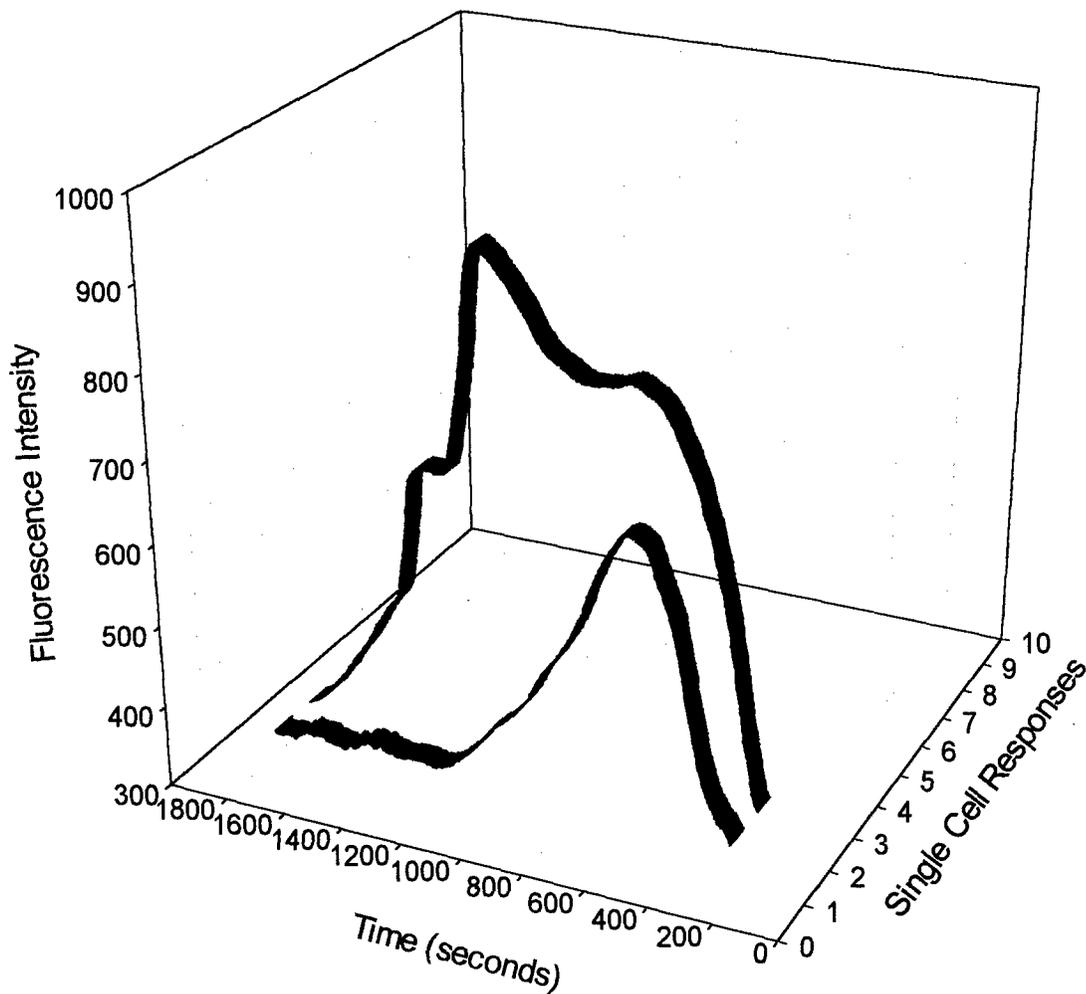
FIGURE 8

MDR CELLS (MCF7-adr) vs DS CELLS (MCF-7)

FIGURE 9

SCHEDULE

SYRINGES	CHAMBER	TIME

B+CalceinAM ✗
B+Hoechst ✗
▮ ✗
BUFFER (B) ✓

$0 < t < 10$

B+CalceinAM ✗
B+Hoechst ✓
▮ ✗
BUFFER (B) ✗

$10 < t < 40$

B+CalceinAM ✓
B+Hoechst ✗
▮ ✗
BUFFER (B) ✗

$40 < t < 70$

B+CalceinAM ✗
B+Hoechst ✗
▮ ✓
BUFFER (B) ✗

$70 < t < 80$

B+CalceinAM ✗
B+Hoechst ✗
▮ ✗
BUFFER (B) ✓

$80 < t < 100$

FIGURE 10

MCF-7 (DS)

MCF-7adr (MDR)
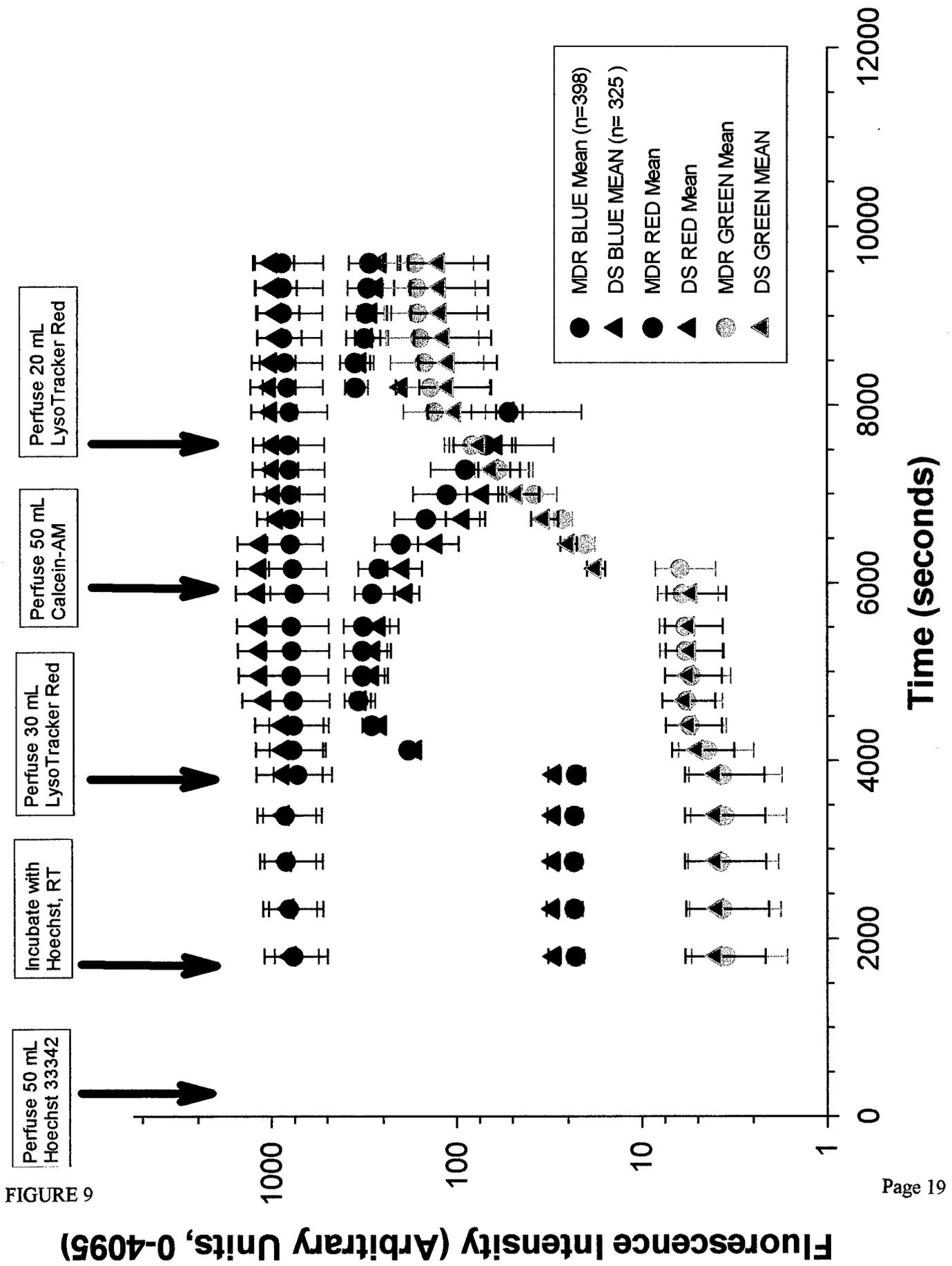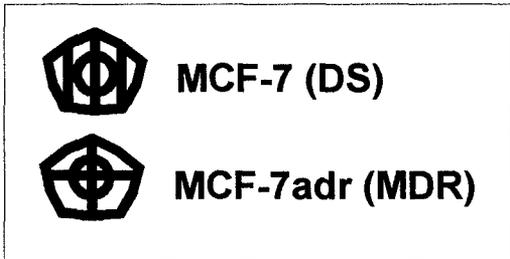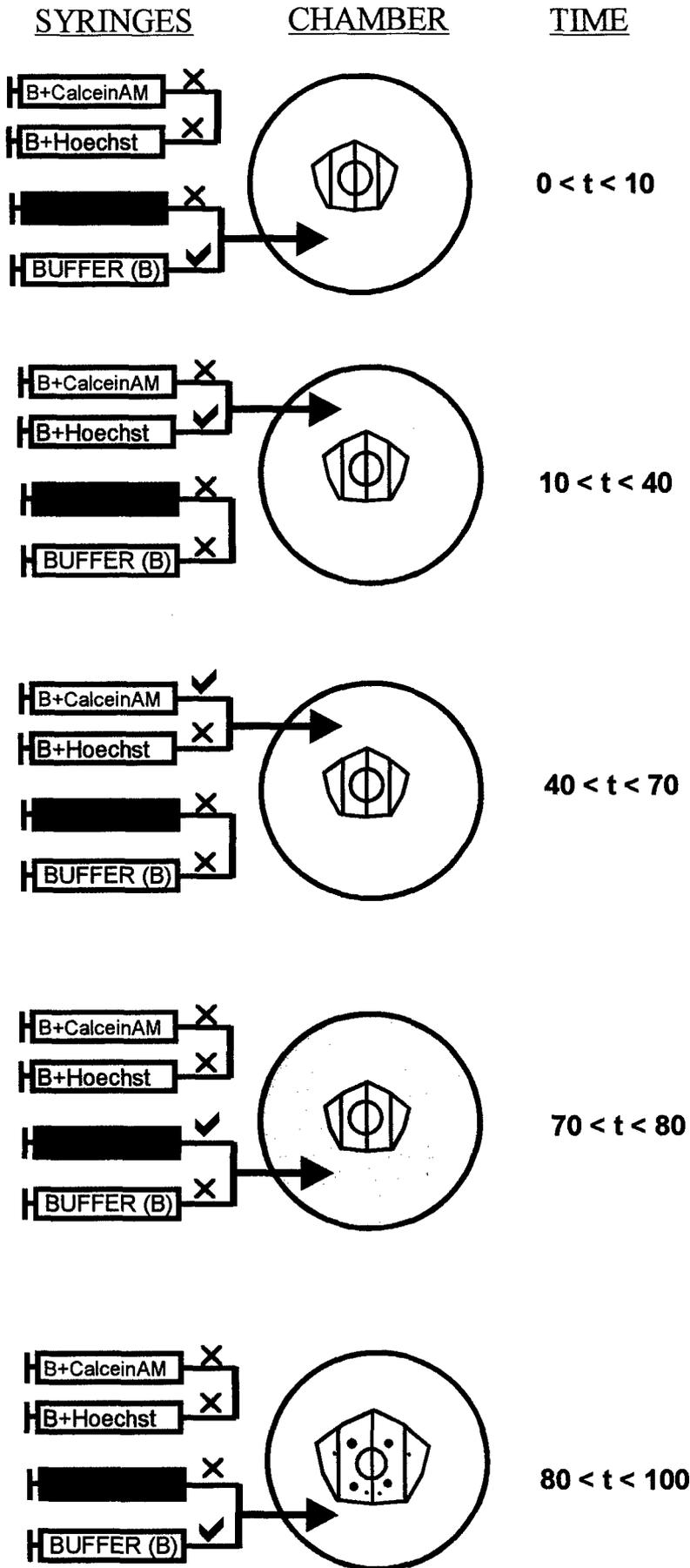
Experimental parameters

Buffer
Syringes (volumes, diameters)
Dyes (concentrations, flow rate)
Modulators
Channels (Exposure Times)
Time Interval(s)
Total Time
Temp
CO_2
Chamber volume
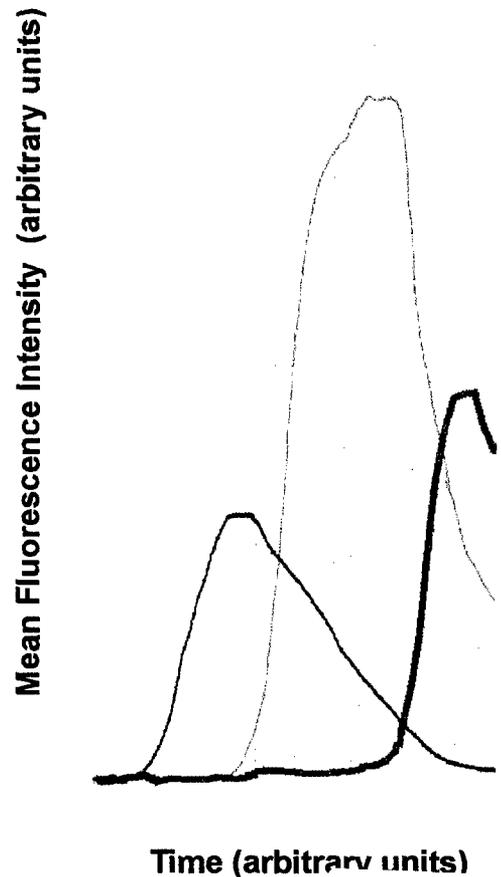
Mean Fluorescence Intensity (arbitrary units)

Time (arbitrary units)

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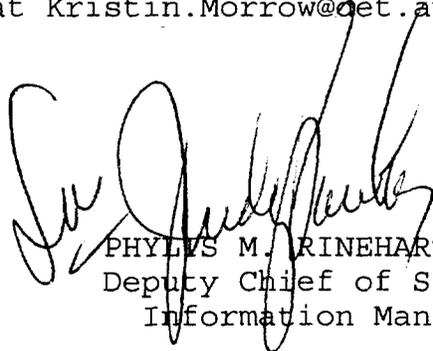
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